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SCREENING AND ISOLATION OF HALOPHILIC BACTERIA PRODUCING INDUSTRIALLY IMPORTANT ENZYMES

Sumit Kumar[1], Ram Karan[1], Sanjay Kapoor[2], S.P. Singh[3], S.K. Khare[1]*

[1]Department of Chemistry, Indian Institute of Technology, Delhi, New Delhi, India; [2]Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi, India; [3]Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India.

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ABSTRACT

Halophiles are excellent sources of enzymes that are not only salt stable but also can withstand and carry out reactions efficiently under extreme conditions. The aim of the study was to isolate and study the diversity among halophilic bacteria producing enzymes of industrial value. Screening of halophiles from various saline habitats of India led to isolation of 108 halophilic bacteria producing industrially important hydrolases (amylases, lipases and proteases). Characterization of 21 potential isolates by morphological, biochemical and 16S rRNA gene analysis found them related to *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera. They belonged to moderately halophilic group of bacteria exhibiting salt requirement in the range of 3-20%. There is significant diversity among halophiles from saline habitats of India. Preliminary characterization of crude hydrolases established them to be active and stable under more than one extreme condition of high salt, pH, temperature and presence of organic solvents. It is concluded that these halophilic isolates are not only diverse in phylogeny but also in their enzyme characteristics. Their enzymes may be potentially useful for catalysis under harsh operational conditions encountered in industrial processes. The solvent stability among halophilic enzymes seems a generic novel feature making them potentially useful in non-aqueous enzymology.

Key words: Halophiles, Biodiversity, Halophilic enzymes, Hydrolases, Solvent-stable.

INTRODUCTION

Screening of new source of novel and industrially useful enzymes is a key research pursuit in enzyme biotechnology. For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc. In this context, the halophiles have emerged as a vast repository of novel enzymes in recent years. Enzymes derived from halophiles are endowed with unique structural features and catalytic power to sustain the metabolic and physiological processes under high salt conditions. Some of these enzymes have been reported to be active and stable under more than one extreme condition (14, 17, 30).

Halophiles have mainly been isolated from saltern

***Corresponding Author. Mailing address:** Enzyme and Microbial Biochemistry Laboratory, Department of Chemistry, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India.; Tel.: +91 11 2659 6533 Fax +91 11 2658 1102.; E-mail: skhare@rocketmail.com / skkhare@chemistry.iitd.ac.in

crystallizer ponds, the Dead Sea, solar lakes and hypersaline lakes (24, 34). Culture dependent diversity studies on halophiles have been done from Tunisian Solar Saltern (4), Tuzkoy salt mine, Turkey (6), Howz Soltan Lake, Iran (28) and hypersaline environments in south Spain (29). In Indian context, the halophilic diversity has been limited to the marine salterns near Bhavnagar (9), Lonar Lake (16) and Peninsular coast (26). Enzymatic diversity among halophiles of Indian origin has hardly been profiled. A systematic study on diversity of halophiles from different saline habitats of India would be of great importance, especially comparison of biochemical, metabolic and enzymatic characteristics among them.

The present study focuses on the (i) isolation of moderate halophiles from various saline habitats of India viz. coastal regions of Gujarat, Goa, Kerala and Sambhar Salt Lake, Rajashthan (ii) screening for industrially important enzymes (especially amylases, lipases and proteases) and (iii) studying novel properties in these enzymes.

MATERIALS AND METHODS

Isolation of enzyme producing halophiles

Soil and water samples were collected from Sambhar Salt Lake (Rajasthan, 26°58′N75°05′E), sea coast of Kozhikode (Kerala, 11°25′N75°77′E), Goa (Goa, 15°59′N73°73′E), Nagoa (Diu, 20°71′N70°92′E), Somnath (Gujarat, 20°88′N70°40′E), Veraval (Gujarat, 20°91′N70°35′E) and Triveni Sangam (Gujarat, 20°71′N70°97′E).

Halophiles were isolated by salt (100.0/200.0 gL^{-1}, NaCl) and substrate (starch/ olive oil/ gelatin) enrichment. The isolation media contained (gL^{-1}): NaCl 100.0/200.0; Starch/ olive oil/ gelatin 10.0; $MgSO_4.7H_2O$ 0.4; $MgCl_2.6H_2O$ 0.7; $CaCl_2.2H_2O$ 0.5; KH_2PO_4 0.3; K_2HPO_4 0.3; $(NH_4)_2SO_4$ 0.5; 0.01% of trace elements solution containing (gL^{-1}) metal: B 0.26; Cu 0.5; Mn 0.5; Mo 0.06 and Zn 0.7 (27). Different dilutions of soil or water samples were added to the above medium and incubated at 30 ºC for 96h. The growth was diluted 10 times and plated on complete medium agar (gL^{-1}): glucose 10.0; peptone 5.0; yeast extract 5.0; KH_2PO_4 5.0; agar 30.0; and NaCl 100.0. Resultant colonies were purified by repeated streaking on complete media agar. The isolates were stored at 4 ºC and sub-cultured at 15 days intervals.

Screening of hydrolase activities

Hydrolase producing bacteria among the isolates were screened by plate assay on starch, tributyrin and gelatin agar plates for amylase, lipase and protease respectively.

Amylolytic activity of the cultures was screened on starch nutrient agar plates containing gL^{-1}: starch 10.0; peptone 5.0; yeast extract 3.0; agar 30.0; NaCl 100.0. The pH was from 7.0 to 10.0 depending on experimental conditions. After incubation at 30 ºC for 72h, the zone of clearance was determined by flooding the plates with iodine solution. The potential amylase producers were selected based on ratio of zone of clearance diameter to colony diameter.

Proteolytic activity of the isolates was similarly screened on gelatin nutrient agar plates containing 10.0 gL^{-1} of gelatin. The isolates showing zones of gelatin clearance upon treatment with acidic mercuric chloride were selected and designated as protease producing bacteria.

Lipase activity of the cultures was screened on tributyrin nutrient agar plates containing 1% (v/v) of tributyrin. Isolates that showed clear zones of tributyrin hydrolysis were identified as lipase producing bacteria.

Identification and characterization of potential halophilic isolates

For identification of strains by 16S rRNA gene sequencing, the genomic DNA was extracted by PrepManTM Ultra sample preparation kit (Applied Biosystems Inc, CA, USA). The 16S rRNA gene sequence was obtained by using MicroSeq® 16S rRNA gene sequencing kit containing universal primers (Applied Biosystems Inc., CA, USA). The identification of phylogenetic neighbours was initially carried out by the BLAST (1) and megaBLAST (35) programmes against the database of type strains with published prokaryotic

names. The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/) (7). The phylogenetic tree was constructed using CLC genomics workbench 4.0.3 (CLC Bio, Denmark).

The biochemical characterization was carried out by HiBio-ID Test Kits.

Production of hydrolases

Hydrolase activities of the isolate exhibiting larger zone of clearance on plate were further confirmed by assaying enzymatic activity in broth. The isolates were seeded into the medium containing (gL^{-1}): gelatin/starch/olive oil 10.0; peptone 5.0; yeast extract 5.0; NaCl 50.0-200.0 and pH 7.0. The incubation was carried out at 150 rpm and 30 °C for 96h. The cells were harvested by centrifugation at 10,000xg for 10 min at 4 °C and cell free supernatant was assayed for respective enzymatic activities.

Enzyme assay

Amylase was assayed following the method of Bernfeld (5) using starch as a substrate. One unit of amylase activity was defined as the amount of enzyme releasing 1 μmol of maltose equivalent per minute from soluble starch under assay conditions. Lipase activity was determined using p-nitrophenol palmitate (pNPP) as substrate following the method described by Kilcawley *et al.* (18). One unit of lipase activity was defined as the amount of enzyme liberating 1 nmol of pNP (p-nitrophenol) per minute under standard assay conditions. Protease activity was measured by using casein as a substrate following the method of Shimogaki *et al*. (31). One unit of protease activity is defined as the amount of enzyme liberating 1 μg of tyrosine per minute under assay conditions.

Characterization of enzymes

pH optimum was determined by assaying the enzyme at various pH ranging from 5.0 to 10.0 using 0.1 M sodium acetate buffer (pH 5.0-5.5), 0.1 M sodium phosphate buffer (pH 6.0-7.5) and 0.1 M Tris-HCl buffer (pH 8.0-10.0).

The effect of salt on enzymatic activity was determined by varying salt concentrations (0-20% NaCl) in the assay mixture. For determining the temperature optima, assay was carried out at various temperatures (30-80 ºC). The thermal stability of enzymes was determined by preincubating at various temperatures (40-80 °C), and estimating the residual activity in aliquots withdrawn at various time intervals. Maximum temperature at which enzyme retained 100% activity for 1h was determined. Other conditions were kept same as per standard assay procedures.

Organic solvent stability

Three millilitres of enzyme was mixed with 1.0 mL of organic solvents in screw capped tubes. The mixture was incubated at 30 ºC with constant shaking at 200 rpm for 24h. Samples were withdrawn from aqueous phase and residual enzyme activity estimated. Enzyme incubated without solvent was treated as control.

All the experiments were done in triplicate and the variation was within ±5%.

RESULTS AND DISCUSSION

Isolation of halophiles and screening of hydrolase producers

Halophiles have been perceived as a potential source of industrially useful enzymes endowed with exceptional stabilities. The present study was undertaken to screen stable enzymes from halophiles occurring in saline habitats of India. The site dependent diversity of halophiles from west coast, south coast and Salt Lake located in western part of India was investigated. A total of 108 halophilic isolates producing hydrolases were isolated by salt (NaCl) and substrate (starch/ olive oil/ gelatin) enrichment.

Distributions of hydrolytic activity among the isolates are summarized in Table 1. Seventy eight isolates secreted only

one predominant enzyme, protease (33), lipase (23) and amylase (22) respectively. Nine isolates produced both lipase and protease, whereas one isolate showed all three activities. Based on ratio of zone of clearance diameter to colony diameter, twenty one isolates were selected for further study.

Table 1. Sampling sites and distribution of hydrolases producing halophiles

Sample site	Sample type	pH	Temperature (°C)	Gram-positive isolates	Gram-negative isolates	Enzymes present		
						Amylase	Lipase	Protease
Kozhikode, Kerala	Sea water and sand	7.6	31.2	3	9	5	5	4
Nagoa, Diu	Sea water and soil	7.9	30.6	9	8	6	7	7
Somnath, Gujarat	Sea water	7.7	30.5	7	11	4	9	8
Triveni Sangam, Gujarat	Sea water and soil	7.8	31.5	9	12	7	5	11
Veraval, Gujarat	Sea water	7.7	30.3	4	5	2	5	5
Goa	Sea water	7.7	30.6	4	7	2	3	8
Sambhar Salt Lake, Rajasthan	Soil and water	7.8	35 .0	11	9	7	4	11

Characterization of halophilic isolates

Among the selected isolates, sixteen were Gram-positive and five Gram-negative. The isolates generally grew in the salt range of 3-20% NaCl, with an optimum requirement of about 5% salt indicating them to be moderately halophilic according to the classification of Kushner (20). The isolates grew in alkaline pH range at average pH~ 7.5-8.0.

Identification by 16S rRNA gene analysis

The 16S rRNA gene analysis related their grouping in nine major genus; *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus*. Gram-negative isolates of *Marinobacter*, *Chromohalobacter* and *Halomonas* genus belonged to *Gamma-Proteobacteria* and Gram-positive isolates of *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Oceanobacillus* and *Bacillus* belonged to *Firmicutes*.

The GenBank accession numbers of the isolates along with their closest phylogenetic neighbour are presented in Table 2. *Geomicrobium* was the most common genus, six halophilic isolates affiliated to this genus showed similarity to *Geomicrobium halophilum*. The genus *Marinobacter* was found only from the Kozhikode. *Virgibacillus* was represented from Kozhikode, Triveni Sangam and Nagoa. The phylogenetic association of the isolates is shown in Figure 1. All the nine genus obtained in present study, are commonly occurring in various saline habitats across the globe. Presence of *Marinobacter*, type isolate *Marinobacter hydrocarbonoclasticus* has been reported from Meditteranean sea (13). *Halobacillus litoralis* (33), *Oceanobacillus iheyensis* (21) and *Geomicrobium halophilum* (10) have been isolated from Great Salt Lake, Iheya ridge and soil samples from Japan respectively.

Table 2. 16S rRNA gene identification of halophilic bacterial isolates producing potent hydrolases

Sample Site	Isolate	GenBank Accession number	Closest phylogenetic neighbour	Similarity (%)
Sambhar Lake	Haloalkaliphilic bacterium EMB1	EU621827	*Geomicrobium halophilum*	99.458
	Haloalkaliphilic bacterium EMB2	EU621828	*Geomicrobium halophilum*	99.457
	Haloalkaliphilic bacterium EMB3	EU621829	*Staphylococcus xylosus*	100
	Haloalkaliphilic bacterium EMB4	EU669822	*Bulleidia extructa*	88.996
Kozhikode	*Marinobacter* sp. EMB5	FJ525429	*Marinobacter litoralis*	99.864
	Marinobacter sp. EMB6	GU059907	*Marinobacter litoralis*	99.863
	Virgibacillus sp. EMB7	GU059906	*Virgibacillus halodenitrificans*	99.414
	Marinobacter sp. EMB8	GU059908	*Marinobacter santoriniensis*	96.288
Goa	*Bacillus* sp. EMB9	GU059909	*Bacillus lehensis*	99.395
	Oceanobacillus sp. EMB10	GU059910	*Oceanobacillus iheyensis*	99.607

Table 2. Continuation.

Triveni Sangam	*Chromohalobacter* sp. EMB12	GU059912	*Chromohalobacter israelensis*	100
	Virgibacillus sp. EMB13	GU059913	*Virgibacillus halodenitrificans*	99.23
	Halobacillus sp. EMB14	GU059914	*Halobacillus kuroshimensis*	99.609
Nagoa	*Halobacillus* sp. EMB15	GU059915	*Halobacillus trueperi*	99.414
	Virgibacillus sp. EMB16	GU059916	*Virgibacillus halodenitrificans*	99.219
	Halobacillus sp. EMB17	GU059917	*Halobacillus kuroshimensis*	99.608
	Haloalkaliphilic bacterium D-10-102	GU059919	*Geomicrobium halophilum*	100
Somnath	*Halomonas* sp. EMB11	GU059911	*Halomonas salina*	99.795
	Haloalkaliphilic bacterium EMB18	GU216650	*Geomicrobium halophilum*	99.796
	Haloalkaliphilic bacterium S-15-9	GU059918	*Geomicrobium halophilum*	100
Veraval	Haloalkaliphilic bacterium Ve2-20-92	GU059920	*Geomicrobium halophilum*	100

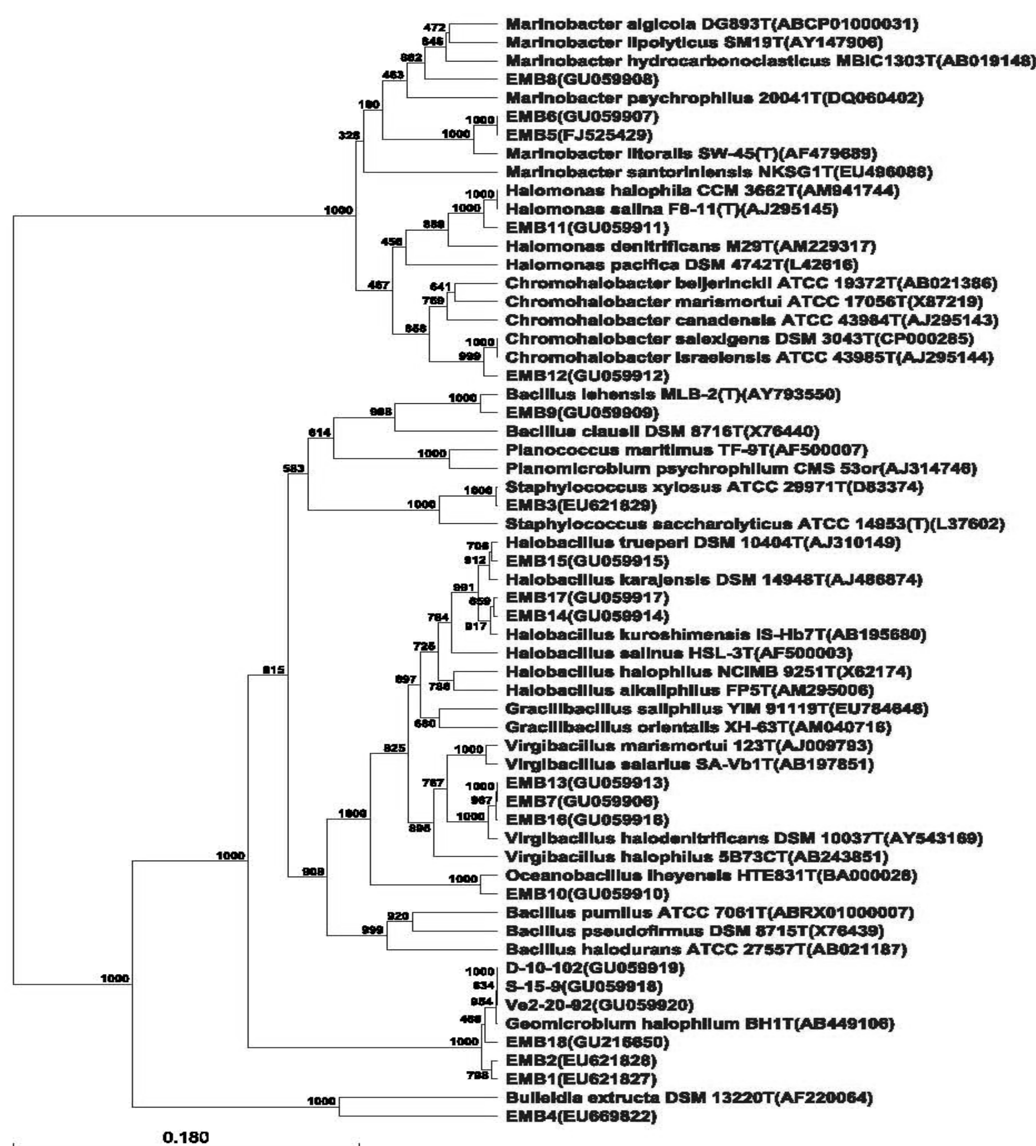

Figure 1. Phylogenetic inference based on 16S rRNA gene analysis of halophilic isolates. Each organism is preceded by its NCBI accession number. Bootstrap values and horizontal scale bar representing number of substitutions per nucleotide are indicated.

Screening and preliminary characterization of the halophilic enzymes

Since the basic objective of study was to screen novel/ stable enzymes among halophiles, the isolates were grown in culture media to obtain crude enzyme for further characterization. Amylase, lipase and protease were chosen for the study, considering their high industrial usage. Maximum amylase was produced by *Marinobacter* sp. EMB8. Lipase and protease production level were maximum in *Marinobacter* sp. EMB5 and *Bacillus* sp. EMB9 respectively.

Crude enzymes were partially characterized to investigate their properties and stability (Table 3). Their optimum salt requirement in most cases was in the range of 1-2%. These were optimally active at pH 9.0 or above, in general. Their temperature optimum ranged between 50-65 °C. Interestingly, all the hydrolases were stable in organic solvents. Based on these results, hydrolases from moderate halophiles can be categorized to be alkaline in nature, moderate in salt requirement and endowed with solvent stability, a common novel feature among all.

Table 3. Preliminary characterization of halophilic enzymes

Sample Site	Microorganism	Potent enzyme produced	Production level (UmL^{-1})	pH optima	Salt optima (%, NaCl)	Temp. optima (°C)	Solvent-stability† (25%, v/v)	Thermal stability (°C) #
Sambhar Lake	Haloalkaliphilic bacterium EMB1	Protease	28	9.5	5	50	Stable	50
	Haloalkaliphilic bacterium EMB2	Protease	37	10.0	3	50	Stable	50
	Haloalkaliphilic bacterium EMB3	Protease	21	9.0	6	45	Stable	50
	Haloalkaliphilic bacterium EMB4	Amylase	1.2	9.0	3	40	Stable	50
Kozhikode	*Marinobacter* sp. EMB5	Lipase	129	9.0	2	50	Stable	60
	Marinobacter sp. EMB6	Lipase	88	9.5	2	65	Stable	60
	Virgibacillus sp. EMB7	Protease	156	7.5	1	60	Stable	50
	Marinobacter sp. EMB8	Amylase	4	7.0	1	45	Stable	50
Goa	*Bacillus* sp. EMB9	Protease	191	9.0	1	60	*	*
	Oceanobacillus sp. EMB10	Protease	26	9.0	1	55	*	*
Triveni Sangam	*Chromohalobacter* sp. EMB12	Lipase	65	9.5	2	65	Stable	50
	Virgibacillus sp. EMB13	Protease	62	7.5	1	60	Stable	50
	Halobacillus sp. EMB14	Amylase	0.3	7.5	1	55	*	*
Nagoa	*Halobacillus* sp. EMB15	Amylase	0.2	8.0	1	55	*	*
	Virgibacillus sp. EMB16	Protease	60	7.5	1	60	*	*
	Halobacillus sp. EMB17	Amylase	0.2	7.5	1	55	*	*
	Haloalkaliphilic bacterium D-10-102	Lipase	7	9.5	1.5	65	*	*
Somnath	*Halomonas* sp. EMB11	Lipase	42	9.0	2	65	*	*
	Haloalkaliphilic bacterium EMB18	Protease	38	10.0	2	55	Stable	55
	Haloalkaliphilic bacterium S-15-9	Lipase	57	9.5	1.5	65	Stable	60
Veraval	Haloalkaliphilic bacterium Ve2-20-92	Lipase	15	9.5	1.5	65	*	*

* Not defined
† Solvent stability checked in hexane, cyclohexane, decane, dodecane and toluene
# Stability for 1h

Prominent amylase, lipase and protease producers have been reported from *Salinivibrio*, *Halomonas* and *Salinivibrio* genus respectively from different hypersaline environment of south Spain (29). Study from Howz Soltan Lake, Iran showed majority of amylase producers were *Oceanobacillus* and *Halobacillus*. *Gracibacillus* and *Halomonas* produced lipase and *Virgibacillus* produced protease (28). The hydrolase producing ability was also observed in moderately halophilic bacteria from *Marinobacter*, *Chromohalobacter* and *Halomonas* genera from the Tunisian Solar Saltern (4).

In the present work, amylase from *Marinobacter* sp. EMB8, *Halobacillus* sp. EMB14, EMB15 and EMB17 and Haloalkaliphilic bacterium EMB4 have shown noticeable production level (up to 4.0 UmL^{-1}). Among halophiles amylase production has been previously reported from *Halomonas meridiana* (8) and *Chromohalobacter* sp. TVSP 101 (25). Amoozegar *et al.* (2) have characterized *Halobacillus* sp. MA-2 amylase, which exhibited pH optima at 7.5-8.5 and temperature optima at 50 ºC. As compared to this, all three isolates of *Halobacillus* sp. although have pH optima in 7.5-8.0 range and temperature optimum in the range 55-60 ºC. Amylase from a *Marinobacter* genus is being reported for the first time to best of our knowledge.

Marinobacter sp. EMB5, *Marinobacter* sp. EMB6, *Halomonas* sp. EMB11, *Chromohalobacter* sp. EMB12 and four *Geomicrobium* sp. isolate were found to be potent lipase producers (up to 129.0 UmL^{-1}). *Marinobacter lipolyticus* (22) and *Chromohalobacter* sp. (29) have been previously described for lipase production. The lipase from *Geomicrobium* sp. is being reported for the first time to best of our knowledge. Till date very few halophilic lipases have been characterized, these are from *Salicola* sp. IC10 (23) and *Salinivibrio* sp. strain SA-2 (3). Solvent stability has not been reported in any these cases, which is highlight of *Geomicrobium* sp. lipase characteristics in present study.

Three isolates belonging to *Virgibacillus* sp., *Geomicrobium* sp. and one each *Oceanobacillus* and *Bacillus* sp. were potent protease producers (up to 191 UmL^{-1}). All the *Virgibacillus* sp. isolates were only protease producers irrespective of site of their isolation. *Virgibacillus* sp. SK33 protease has been reported to be salt tolerant and stable in organic solvents (32).

Solvent stability a generic novel feature in halophiles

Solvent stability as a generic feature marks these novel enzymes for further study. Solvent stability has been observed as a novel trait among the halophilic enzymes in recent years (15). Solvent stability in halophilic enzymes may be attributed to the fact that they work in low water activity caused by high salt surroundings. Such enzymes are potentially useful in synthesis applications (12). Halophilic hydrolases investigated in present study were stable in 25% (v/v) concentration of hexane, cyclohexane, decane, dodecane and toluene.

Amylase from *Haloarcula* sp. strain S-1 remained active and stable in presence of 66% benzene, toluene and chloroform (11). Solvent stable proteases have been reported from *Halobacterium salinarum* (19) and *Geomicrobium* sp. EMB2 (17).

All the 108 hydrolase producers isolated from diverse saline habitats of India belong to moderate halophiles category. Moderately halophilic bacteria constitute the most versatile group of microorganisms that could be used as a source of salt-adapted enzymes. These have advantage over extreme halophiles in that they do not have a strict salt requirement and grow in wide salt range. Solvent stability of amylase, lipase and protease from these halophilic isolates make them potentially useful for application in non-aqueous enzymology for synthesis of oligosaccharides, esters and peptides.

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